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Temporal analysis of the early BMP functions identifies distinct anti-organizer and mesoderm patterning phases

Karen Marom, Vered Levy, Graciela Pillemer, Abraham Fainsod*

Department of Cellular Biochemistry and Human Genetics, Faculty of Medicine, Hebrew University, POB 12272, Jerusalem 91120, Israel

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Abstract

BMP signaling performs multiple important roles during early embryogenesis. Signaling through the BMP pathway is mediated by different BMP ligands expressed in partially overlapping temporal and spatial patterns. Assignment of different BMP-dependent activities to the individual ligands has relied on the patterns of expression of the various *BMP* genes. Temporal analysis of BMP signaling prior to and during gastrulation was performed using glucocorticoid-controlled Smad proteins. Overexpression of the BMP-specific Smad1 and Smad5 revealed that suppression of Spemann's organizer formation in *Xenopus* embryos can only take place by activating the BMP pathway prior to the onset of gastrulation. Blocking BMP signaling with the inhibitory Smad, Smad6, results in dorsalized embryos or secondary axis induction, only when activated up to early gastrula stages. *BMP2* efficiently represses organizer-specific transcription from the midblastula transition onwards while *BMP4* is unable to prevent the early activation of organizer-specific genes. Manipulation of the BMP pathway during mid/late gastrula affects mesodermal patterning with no external phenotypic effects. These observations suggest that the malformations resulting from inhibition or promotion of organizer formation, ventralized or dorsalized, respectively, are the result of a very early BMP function, through its antagonism of organizer formation. This function is apparently fulfilled by BMP2 and only at its latest phase by BMP4. Subsequently, BMP functions in the patterning of the mesoderm with no apparent phenotypic effects.

Keywords: Spemann's organizer; Smad proteins; Mesodermal patterning; *BMP2*; *BMP4*; Gastrula; *Xenopus*; Blastula

Introduction

In *Xenopus* embryos, mesoderm is initially specified during pregastrula stages along the equatorial region, the marginal zone (reviewed in Harland and Gerhart, 1997; Heasman, 1997). This process involves the integration of numerous signals in order to induce and achieve early patterning of the mesoderm (Schohl and Fagotto, 2002). One of the signals important for this process involves TGFβ factors of the bone morphogenetic protein family (BMP; Graff, 1997; Harland and Gerhart, 1997; Heasman, 1997; Hogan, 1996; Sidi et al., 2003). These factors perform numerous functions during embryogenesis among them; antagonism of Spemann's organizer formation, promotion of ventral mesodermal fates, inhibition of neural differentiation, patterning of the ectoderm and inhibition of endodermal differentiation. During blastula and early gastrula, four BMP factors are expressed, *BMP2*, *BMP4*, *BMP7*, and *Gdf6*, but the detailed function(s) of each have not been determined (Chang and Hemmati-Brivanlou, 1999; Clement et al., 1995; Fainsod et al., 1994; Hawley et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995; Wawersik et al., 2005). Genetic analysis of the *BMP* genes was undertaken in mice and zebrafish. Although striking phenotypes were observed, assignment of specific roles to distinct members of this family has proven difficult (Dudley and Robertson, 1997; Hawley et al., 1995; Kishimoto et al., 1997; Schmid et al., 2000; Winnier et al., 1995; Zhang and Bradley, 1996).

In early frog embryos, two BMP ligands, BMP2 and BMP7, are present as strong maternal contributions (Hemmati-Brivanlou and Thomsen, 1995; Nishimatsu et al., 1992), while strong *BMP4* expression appears zygotically

* Corresponding author. Fax: +972 2 6415848.
E-mail address: fainsod@cc.huji.ac.il (A. Fainsod).

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towards the onset of gastrulation (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995) and *Gdf6* even later (Chang and Hemmati-Brivanlou, 1999). *BMP2* or *BMP4* overexpression results in reduction of dorsal and anterior structures (Clement et al., 1995; Dale et al., 1992; Hemmati-Brivanlou and Thomsen, 1995; Jones et al., 1992), "ventralized" embryos (Kao and Elinson, 1988), and reduction of BMP4 activity can rescue UV-ventralized embryos (Steinbeisser et al., 1995).

One of the embryonic structures important for normal embryonic development and patterning of the mesoderm is the embryonic organizer, Spemann's organizer in amphibia (Harland and Gerhart, 1997). The organizer is established in *Xenopus* embryos on the future dorsal side by an inductive signal involving the Wnt signaling pathway originating from dorso-vegetal cells (Harland and Gerhart, 1997). Spemann's organizer is responsible for signals promoting dorsal patterning of the mesoderm and neural induction (De Robertis et al., 2000; Harland and Gerhart, 1997; Sasai and De Robertis, 1997; Schoenwolf and Smith, 2000; Sive, 1993). Among the signals secreted by the organizer are a number of BMP signal antagonists like *noggin* (Zimmerman et al., 1996), *chordin* (Piccolo et al., 1996), and *follistatin* (Fainsod et al., 1997). Also, transcriptional inhibitors of BMP downstream genes are expressed in the organizer (Ferreiro et al., 1998; Yao and Kessler, 2001). Inhibition of BMP signaling by the organizer is important for neural induction and dorsoventral patterning of the mesoderm (De Robertis et al., 2000; Fainsod et al., 1994; Sasai et al., 1995; Steinbeisser et al., 1995). In parallel, BMP downstream genes have been shown to function as efficient repressors of organizer-specific gene expression (Melby et al., 1999; Onichtchouk et al., 1998; Shapira et al., 2000). The mutually repressive interactions between the dorsal (organizer) and ventral (BMP) signals and their relative strengths determine the dorsoventral patterning of the embryo (De Robertis and Kuroda, 2004).

BMP signaling involves ligand binding to an heterodimeric receptor leading to phosphorylation events that activate the regulatory Smad protein (R-Smad) (reviewed in Massague and Wotton, 2000; Shi and Massague, 2003; Ten Dijke et al., 2000). The BMP pathway involves additional Smad proteins, including the common partner (Co-Smad) and inhibitory-Smads (I-Smad), that mediate and regulate the intracellular signaling. In BMP signaling, the pathway-specific R-Smads: Smad1, Smad5, and Smad8 (Graff et al., 1996; Nakayama et al., 1998; Suzuki et al., 1997), are activated by phosphorylation. Overexpression of these R-Smads results in developmental malformations usually attributed to over-activation of BMP signaling. Two Smad proteins are known to function as I-Smads in BMP signaling: Smad6 and Smad7. *Smad6* overexpression in *Xenopus* embryos specifically blocks the BMP signaling pathway (Hata et al., 1998).

To better understand the role(s) of BMP signaling in the early embryo, temporal and spatial localization of endogenous phosphorylated BMP-specific R-Smads has been performed showing early widespread activation of the pathway followed by ventral–lateral localization during gastrula stages (Faure et al., 2000; Kurata et al., 2001; Schohl and Fagotto, 2002). BMP overexpression can suppress the formation of Spemann's organizer and promote ventral mesodermal tissues at the expense of dorsal/lateral ones. Therefore, in the present study, we aimed at clearly defining the anti-organizer and mesoderm patterning phases of BMP signaling. With the aid of BMP2 and BMP4 overexpression and hormone-controlled BMP-specific R- and I-Smad constructs, we studied the different roles of BMP signaling as a function of time during blastula and gastrula stages. We were able to define temporally restricted responses of marginal zone cells to BMP signals at different developmental stages. We show that BMP-dependent embryonic malformations, the "ventralized" phenotype, can only be induced by activation of BMP signaling during blastula up to early gastrula. This phenotype is the result of an organizer inhibitory function of BMP and it is apparently fulfilled by BMP2 in the *Xenopus* embryo. During gastrula stages, manipulation of BMP signaling results in mesodermal patterning defects with no overt external developmental malformations, and this activity can be attributed to BMP4. A model is proposed where during late blastula/early gastrula BMP signaling, maternal BMP2 functions to antagonize the formation of ectopic organizers while during gastrula, zygotic BMP4 functions in dorsal–ventral patterning of the mesoderm.

Materials and methods

Inducible protein chimeras and DNA constructs

To generate Smad1/GR, an internal fragment was amplified from Xmad—pCS2 (Thomsen, 1996), mutating the stop codon to a *Kpn*I site and adding an *Xho*I site using the primers: up 5′CAAGATATCAATAGAGCAGA3′; down 5′TACTCGAGGTACCAGAGACAGAGGAGATGGG3′. This PCR fragment was used to replace the wild type sequences. Subsequently, the GR domain (amino acids 512–777 of the human glucocorticoid receptor; Kolm and Sive, 1995) was fused in frame.

For Smad5/GR, the whole coding sequence from mSmad5-pSP64T (Suzuki et al., 1997) was PCR amplified introducing an upstream ClaI site, and replacing the stop codon by a *Kpn*I site followed by a *Xho*I site. The primers used were: up 5′ATGTATATCGATGACGTCAATGGCCAGCTT3′; down 5′TCCTCGAGGTACCTGAAACAGAAGAAATGGG3′. After subcloning into pCS2, the GR domain including upstream and downstream *Kpn*I sites, was fused in frame.

A fragment of the human *Smad6* in the hSmad6-pcs105 plasmid (Hata et al., 1998) was amplified introducing an upstream *Bam*HI restriction site, and replaced the stop codon by a *Kpn*I restriction site proceeded by an *Xho*I site.

The primers used were: up 5′TGGATCCGGACCATGCCCGAATCTCCGCCAC3′; down 5′TCTCGAGGTACCAAATCTGGGGTTGTTGAGG3′. The *Bam*HI–*Xho*I PCR fragment was cloned into pCS2. Subsequently, a *Bam*HI–*Bsp*EI fragment was cloned from the original hSmad6-pcs105 construct into the plasmid, and finally the GR domain was fused in frame. All clones produced were subjected to sequencing analysis to rule out the possibility of PCR-caused mutations.

The *BMP2* cDNA clone (Nishimatsu et al., 1992) was subcloned into pCS2 to prepare capped RNA after linearization with *Not*I. *BMP4*-capped RNA was prepared as previously described (Fainsod et al., 1994).

Embryos and microinjection

RNA for microinjection was prepared using the RiboMax kit (Promega), and adding cap analog (Roche, Pharmacia) at a ratio of 1:5 (cap analog:GTP). Microinjection of the embryos was performed in 4-cell stage embryos. Embryos were injected in 1× MBSH buffer, and raised to the desired stage in 0.1× MBSH buffer.

In situ hybridization and probes

Embryos at the desired stage were fixed in MEMFA and processed for in situ hybridization, as described (Fainsod et al., 1994). Digoxigenin (Dig)-labeled RNA probes were in vitro transcribed using the RiboMax kit (Promega), and the dig RNA labeling mix (Roche).

The probes used in the in situ hybridization procedure were: *gsc*, the H7 clone (Cho et al., 1991); *Otx2*, the pXOT30 clone (Smith et al., 1993); *Xnot2*, the E14 clone (Gont et al., 1993); *Xwnt8*, the XP5 (pGEM-Xwnt8) clone (Christian et al., 1991); *muscle actin*, the pAC100 clone (Hemmati-Brivanlou et al., 1990); *myoD*, the p3 (pbsKS$^+$-XMyoD) clone (Frank and Harland, 1991); *collagen typeII*, the p7XK500 clone (Bieker and Yazdani-Buicky, 1992); *XCG1*, the H8-1 (pVZ1 plasmid) clone (Sive et al., 1989); *N-CAM*, cloned in the pbsKS$^-$ plasmid (Krieg et al., 1989).

Results

BMP antagonizes organizer formation until the onset of gastrulation

To analyze the developmental roles of BMP signaling manipulation as a function of time, either inhibition or activation of this pathway at different developmental stages was performed. For this purpose, glucocorticoid-controlled inducible versions of the BMP-specific R-Smads, Smad1 (Graff et al., 1996) and Smad5 (Suzuki et al., 1997), and the I-Smad, Smad6 (Hata et al., 1998), proteins were constructed. The Smad proteins were rendered hormone-inducible by fusing them to the hormone-binding domain of the glucocorticoid receptor (GR) and activated by dexamethasone exposure (dex; Kolm and Sive, 1995). As a control, the fusion proteins were activated with dex soon after injection and were compared to embryos injected with mRNA encoding the wild type proteins. The morphological malformations caused by overexpression of the wild type or hormone-regulated proteins were indistinguishable (not shown, see also Fig. 1).

For temporal analysis of the BMP signal, injected embryos were treated with dex at different stages between midblastula and late gastrula and subjected to morphological analysis at stages 29–31 (Nieuwkoop and Faber, 1975). The extent of "ventralization" of the samples was determined by establishing the dorsoanterior index of the samples (DAI; Kao and Elinson, 1988) Activation of Smad1/GR close to midblastula transition (MBT; st. 8) resulted in malformed embryos, lacking head and axial structures, "ventralized" embryos (DAI = 1.8, n = 156; Fig. 1A). Ectopic activation of the BMP pathway at st. 9 resulted also in strong malformations (DAI = 2.6, n = 188; Fig. 1B). At the onset of gastrulation, Smad1/GR activation resulted in weaker defects (DAI = 3.3, n = 129; Fig. 1C). Activation during subsequent gastrula stages resulted in a gradual decrease in the extent of morphological malformations, while weak "ventralization" could be observed by activation at mid/late gastrula (st. 11.5; DAI = 3.9, n = 164; Figs. 1D,E). These results suggest that induction of the "ventralized" phenotype by BMP overexpression is restricted to ectopic activation of the signaling pathway up to early gastrulation.

The Smad5/GR fusion protein was used to corroborate the observations obtained with Smad1. Smad5 activation during late blastula and early gastrula (st. 8–10) resulted in "ventralized" embryos (DAI = 2.6, n = 145; Figs. 1G–H). As observed with the Smad1 construct, activation of the Smad5 fusion protein lost its malformation-inducing capacity with later activation during gastrulation (DAI = 4.3, n = 62; Figs. 1J,K). Our results indicate that the competence to respond to BMP signaling, as manifested by "ventralization" of the embryo, ends during early gastrula stages.

More detailed quantitative analysis of the "ventralization" induced as a function of the developmental stage of activation was performed by analyzing the distribution of DAI in each sample. Activation of the Smad1/GR protein at st. 7 gave rise to the majority of the embryos exhibiting strong ventralization (52%; DAI 0–1) or anterior developmental defects (15%; DAI 2–3; Fig. 2A). The fraction of embryos with DAI of 0–1 decreased towards the onset of gastrulation (st. 10; 18%) and reached background levels by early gastrula (st. 10.5; 7%). Also, the proportion of mildly affected embryos decreased towards early gastrulation and these changes were accompanied by an increase in the percentage of normal or almost normal looking embryos (Fig. 2A). Under these experimental conditions, the Smad1/GR protein had almost no activity in the absence of dex (Fig. 2A). In *Smad5* overexpressing embryos, the extent of ventralization was weaker but the overall temporal effect was the same as with Smad1. Smad5/GR, like Smad1/GR,

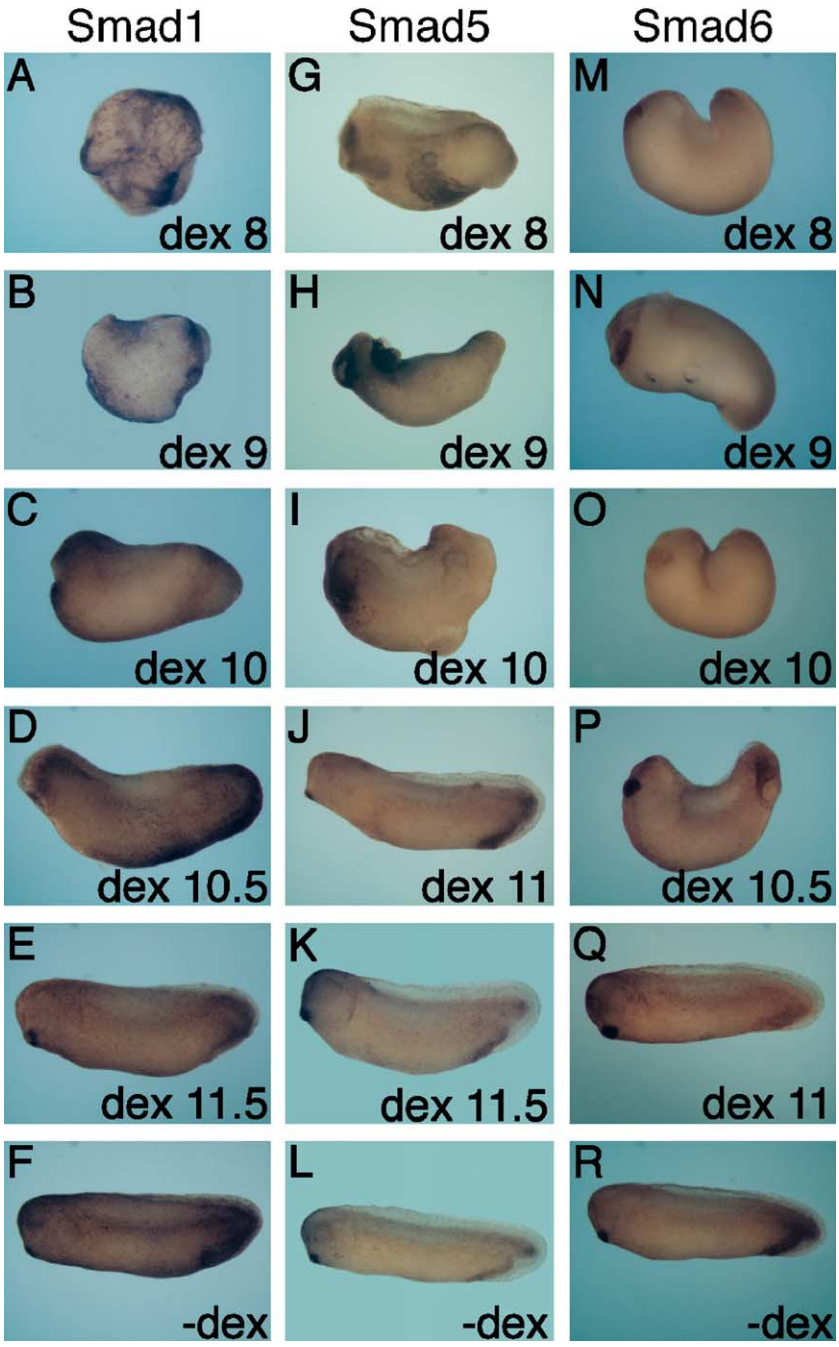

Fig. 1. Timed activation or repression of the BMP signaling pathway during early embryogenesis. Embryos were injected with mRNA encoding the Smad1/GR (A–F), Smad5/GR (G–L), or Smad6/GR (M–R) proteins. The embryos were exposed to dex at the stages marked and incubated to st. 29–31 for phenotypic analysis.

induced the strongest ventralization of the embryos when activated prior to gastrulation (Fig. 2B). Activation of the BMP signaling pathway by *Smad5* overexpression from early gastrula onwards resulted in a small fraction of mildly affected embryos. The quantitative analysis supports the observation that induction of strong anterior–dorsal defects, "ventralization", is the result of ectopic BMP signaling activity during blastula up to early gastrula.

A more direct analysis of the endogenous activity of BMP signaling at these stages requires a loss-of-function approach. The BMP-specific I-Smad, Smad6, was used to specifically block the BMP pathway at different developmental stages. Blocking BMP signaling during blastula and early gastrula resulted in malformed embryos with enhanced dorsal–anterior regions, "dorsalized" embryos (DAI = 5.6, n = 206; Figs. 1M–O). Activation of Smad6/GR from mid-gastrulation onwards did not result in overt morphological defects (DAI = 5.1, n = 40; Fig. 1Q). These observations again supported the suggestion that BMP-dependent dorsalization acts upon an embryonic process

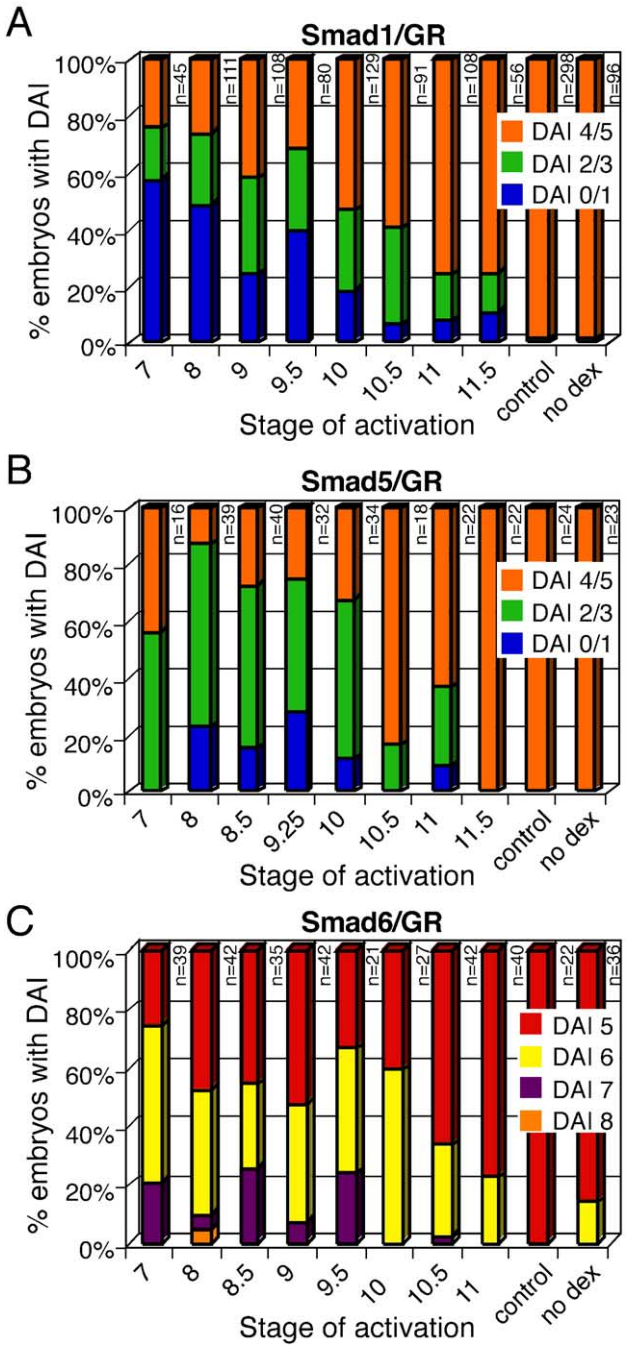

Fig. 2. Dorsoanterior index analysis of activation or repression of the BMP signaling pathway at different developmental stages. The dorsoanterior index (DAI) of embryos injected with Samd1/GR (A), Smad5/GR (B), or Smad6/GR (C) capped RNA and activated at different developmental stages was determined.

taking place during a restricted developmental period from blastula to early gastrula. The BMP blocking effect was also studied by determining the distribution of the DIA in the samples. Dorsalization of the embryos, DAI 6 and 7, was achieved with high efficiency as a result of activation of the chimeric protein from MBT to the onset of gastrulation (Fig. 2C). From early gastrula onwards, the fraction of dorsally enhanced embryos decreased. These results show that "dorsalization" exhibits the same temporal competence as ectopic activation of the BMP signaling pathway that leads to "ventralization" of the embryos. The results suggest an early function for BMP signaling in restricting dorsalization of the embryo by expanded dorsal–anterior embryonic structures.

BMP regulates the formation of Spemann's organizer until the onset of gastrulation

Blocking BMP signaling throughout the embryo results in a dorsalized phenotype while localized inhibition of this pathway in the prospective ventral mesoderm region results in the establishment of an ectopic organizer and formation of secondary axes (Graff et al., 1994; Hawley et al., 1995; Suzuki et al., 1994). The secondary axis assay by localized inhibition of BMP signaling in the ventral marginal zone suggests an inhibitory role on an organizer-inducing activity present there. Therefore, taking advantage of the inducible Smad6 protein, we studied the developmental window during which inhibition of BMP signaling results in efficient induction of secondary axes. Embryos injected ventrally were treated with dex at different developmental stages, allowed to develop to stage 27 and then processed for in situ hybridization with a combined probe mix for neural tube (N-CAM; Krieg et al., 1989), somites (muscle actin; Hemmati-Brivanlou et al., 1990), and cement gland (XCG1; Sive et al., 1989) for secondary axis scoring. Activation of the Smad6/GR protein prior to gastrulation resulted in efficient induction of secondary axes (Figs. 3A,B; st. 8, 55%; st. 9, 39%). A dramatic decrease in secondary axis induction was observed when the BMP pathway was blocked ventrally following the onset of gastrulation (Figs. 3C,D; $\leq$9%). These results show that the developmental window for secondary axis induction via BMP signaling inhibition is the same as that observed for "dorsalization" or "ventralization" of *Xenopus* embryos by manipulation of the BMP signal, suggesting that the malformations observed are the result of interference with normal organizer formation.

The dorsal marginal zone is the normal site of organizer formation (Harland and Gerhart, 1997), and when explanted, it gives rise to dorsal–anterior structures (Fig. 4A). The temporal manipulation of BMP signaling suggested an early effect of this signaling pathway on the formation and early function of the organizer. To further support this observation, we studied the effect of ectopic BMP activity on the differentiation of dorsal marginal zone explants. Embryos injected radially with Smad1/GR were activated at different stages, and dorsal marginal zones were explanted at the onset of gastrulation. For early activation of the fusion protein, the dorsal lips were explanted in buffer containing dex. Injected explants in the absence of dex developed as anterior and dorsal tissues with enlarged head structures and cement glands (Fig. 4A). Activation of the BMP pathway during late blastula (st. 9.5), resulted in the ventralization of

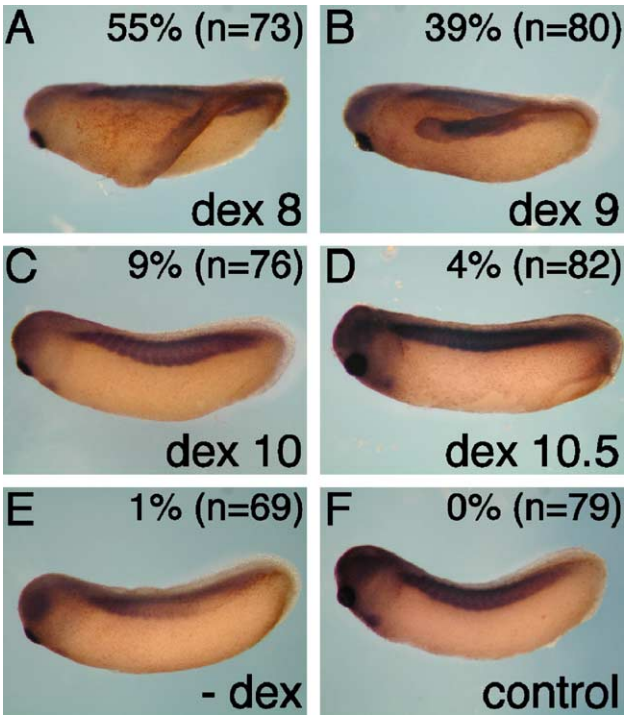

Fig. 3. Secondary axis induction by BMP signaling inhibition as a function of time. Embryos were injected ventrally with Smad6/GR mRNA to induce the formation of secondary axes (A–F). The fusion protein was activated by dex at several developmental stages and the embryos were incubated until st. 27 for analysis. Identification of the secondary axes was achieved by in situ hybridization with probes for *N-CAM* (neural tube), *muscle actin* (somites), and *XCG1* (cement gland). The numbers summarize the percentage of embryos with secondary axes and the sample size.

the dorsal marginal zone explants and prevented the formation of anterior head structures (Fig. 4B). By early gastrula (st. 10.5), activation of BMP signaling induced a weaker ventralization of the dorsal marginal zones where a trunk is evident but they lack head structures (Fig. 4C). Dex treatment during mid/late gastrula stages did not interfere with the development of the explants as anterior head structures (Fig. 4D). These results further support the suggestion that early BMP signaling antagonizes the formation of the embryonic organizer during late blastula up to early gastrula.

Inhibition of organizer-specific gene expression by BMP signaling as a function of time

Molecular analysis of the stage-dependent activation of BMP signaling was performed by studying the changes in expression patterns of organizer-specific genes. Embryos were injected radially at the 4-cell stage with mRNA encoding the Smad1/GR fusion protein. At the desired developmental stages, the embryos were treated with dex and incubated for 4 h prior to subjecting them to in situ hybridization analysis. Ectopic activation of the BMP pathway during late blastula results in the elimination of *gsc* expression (Fig. 5B). Analysis of embryos incubated for longer times following activation (>4 h) did not show recovery from the *gsc* repression (not shown). Activation of the Smad1 protein during early gastrula resulted in a weaker inhibitory effect on *gsc* expression in a smaller fraction of the embryos (Fig. 5D). Ectopic BMP signaling during mid- and late gastrulation had no marked effect on *gsc* expression (Fig. 5F). These results suggest that *gsc* is under negative regulation by BMP signaling during blastula and early gastrula.

Otx2 expression is down-regulated by ectopic BMP signaling only when activated during late blastula stages (Fig. 5H). Activation of BMP signaling by *Smad1* over-expression during gastrula stages had no marked effect on the *Otx2* pattern of expression (Figs. 5J,L). In the case of *Otx2*, it appears that the effect of BMP signaling is more restricted in time.

Xnot2 is expressed in the organizer and the forming notochord (Gont et al., 1993). Ectopic activation of BMP signaling during late blastula resulted in the down-regulation of *Xnot2* in the cells closest to the blastopore lip (Fig. 5N). Activation of the Smad1/GR protein during gastrulation gave rise to an abnormal and weaker pattern of *Xnot2* expression along the dorsal midline (Figs. 5P,R). *Xnot2*, a marker of the dorsal-most type of mesoderm, notochord, appears to be under regulation of the BMP signaling pathway during blastula and gastrula stages. These results suggest that BMP signaling down-regulates most efficiently organizer-specific gene expression during blastula and early gastrula, probably due to its antagonism of organizer formation; at later stages, it seems to affect genes active in mesoderm patterning like *Xnot2*.

BMP activity during mid–late gastrulation

The results presented show that during blastula and early gastrula stages, BMP signaling can regulate the establish-

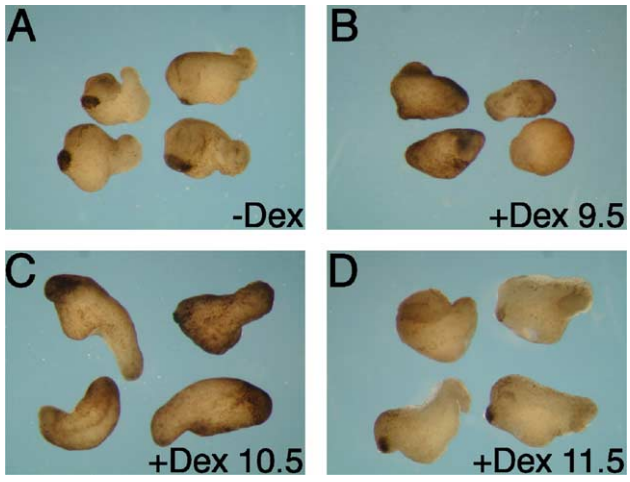

Fig. 4. Temporal effect of BMP signaling on explanted dorsal marginal zones. Embryos were injected with Smad1/GR mRNA and activated by dex treatment at different developmental stages (A–D). At the onset of gastrulation, the dorsal marginal zone were explanted and incubated to st. 30.

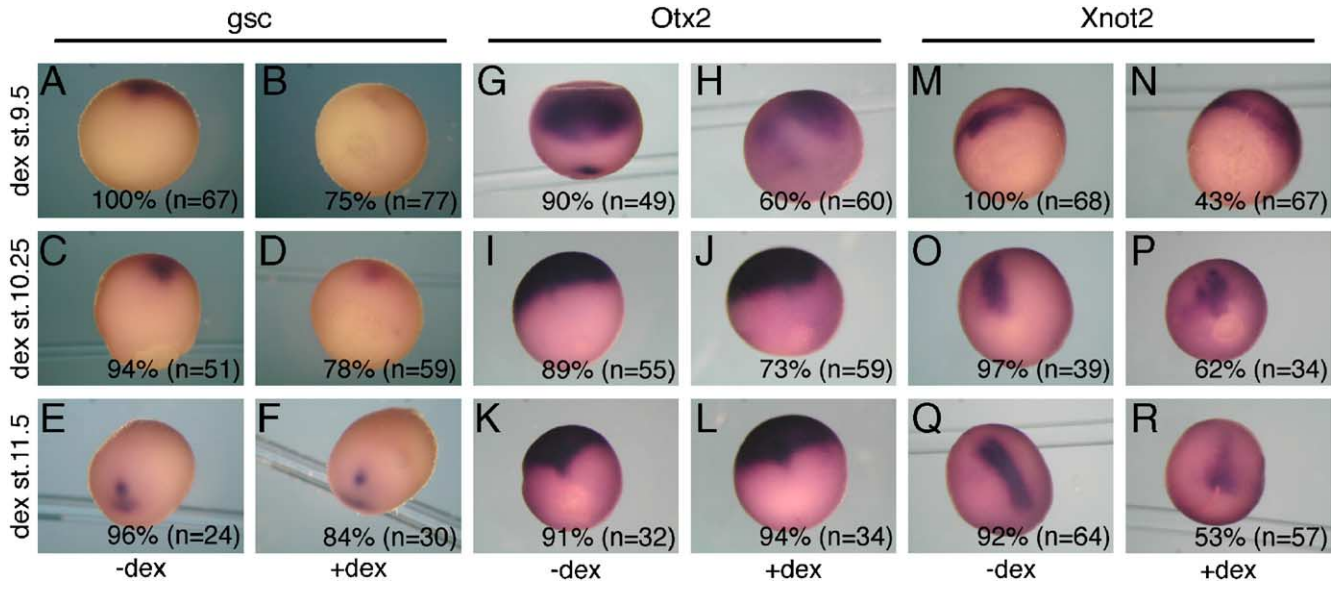

Fig. 5. Inhibition of organizer-specific gene expression by BMP signaling as a function of time. Embryos were injected with Smad1/GR mRNA and treated with dex at the specified stages. Four hours after the dex treatment was initiated, the embryos were fixed and processed for in situ hybridization with *gsc* (A–F), *Otx2* (G–L), and *Xnot2* (M–R) specific probes. The numbers summarize the percentage of embryos resembling the representative embryo and the sample size.

ment of the organizer fate. Interestingly, no external phenotypic effects could be observed by manipulation of the BMP signaling pathway after the onset of gastrulation. On the other hand, BMP4 has been proposed to function during gastrulation as part of the dorsal–ventral network active in mesoderm patterning (Jones et al., 1996). Taking advantage that BMP4 regulates the expression of *Xwnt8* and *MyoD* in a concentration- and time-dependent manner (Hoppler and Moon, 1998; Marom et al., 1999), we studied the role of BMP signaling from mid-gastrulation onwards. Embryos injected with mRNA encoding Smad1/GR or Smad6/GR were treated with dex at st. 11 or 11.5 and analyzed at st. 11.5 or 12, respectively, for *Xwnt8* (Christian et al., 1991) and *MyoD* (Frank and Harland, 1991) expression. Smad1 overexpression during mid-gastrula resulted in elimination of the normal ventral marginal zone expression of *Xwnt8* (Fig. 6B). This pattern resembles a slightly more advanced developmental stage in the normal *Xwnt8* expression attributed to *BMP4*-dependent repression (Fig. 6C; Marom et al., 1999). Blocking the BMP signaling pathway by Smad6 activation during gastrula was able to restore *Xwnt8* transcription to the ventral marginal zone in late gastrula embryos (Fig. 6D), resembling an earlier developmental stage (Fig. 6A). Ectopic activation of the BMP signaling pathway resulted in down-regulation of *MyoD* expression in the dorsal–lateral region of the embryo (Fig. 6F). On the other hand, inhibition of BMP signaling during late gastrula resulted in ectopic *MyoD* expression in regions lateral and ventral to the closing blastopore (Fig. 6H). The late effects of R- and I-Smad overexpression support a role for BMP signaling in the dorsal–ventral patterning of the mesoderm during mid- and late gastrulation.

BMP2 as a negative regulator of organizer formation

BMP4 has been shown to function during gastrula stages primarily in the dorsoventral patterning of the marginal zone (Jones et al., 1996). Activation of organizer-specific genes occurs normally in *BMP4* overexpressing embryos, but transcript levels rapidly decline with the onset of gastrulation (Fainsod et al., 1994; Jones et al., 1996). Temporal analysis of BMP expression during late blastula has shown that, maternal *BMP2* transcripts are apparently replaced by zygotic *BMP4* transcription before the onset of gastrulation (Hemmati-Brivanlou and Thomsen, 1995; Fig. 7). In order to study the inhibitory effect of BMP signaling on organizer formation, we compared the effect of over-expressing *BMP2* and *BMP4* on organizer-specific gene expression. To be able to compare embryos injected with *BMP2* and *BMP4* mRNA, we titrated the RNAs to induce a similar DAI of the samples. Embryos injected with *BMP4* or *BMP2* mRNA at concentrations that induce a DAI of 2 or 3 were subjected to in situ hybridization analysis at stage 9.5 to determine the pattern of expression of *gsc*. By late blastula, BMP2 (DAI = 2.1) efficiently repressed the expression of the organizer gene *gsc* (77%, n = 37; Fig. 8B) and at DAI = 3.1 inhibited *gsc* expression in 60% of the embryos (n = 33). BMP4 had a limited repressive effect on *gsc* at DAI = 2 (14.3%, n = 35; Fig. 8C) and at DAI = 3.2 (20%, n = 37). Similar results were obtained when *Otx2* expression was studied after *BMP2* and *BMP4* over-expression (Figs. 8H,I). By mid-gastrula stages, the same amounts of BMP encoding mRNAs efficiently repressed the expression of organizer/dorsal genes (Figs. 8E,F,K,L). Similar results were obtained when an ectopic organizer was induced by ectopic expression of the organizer-

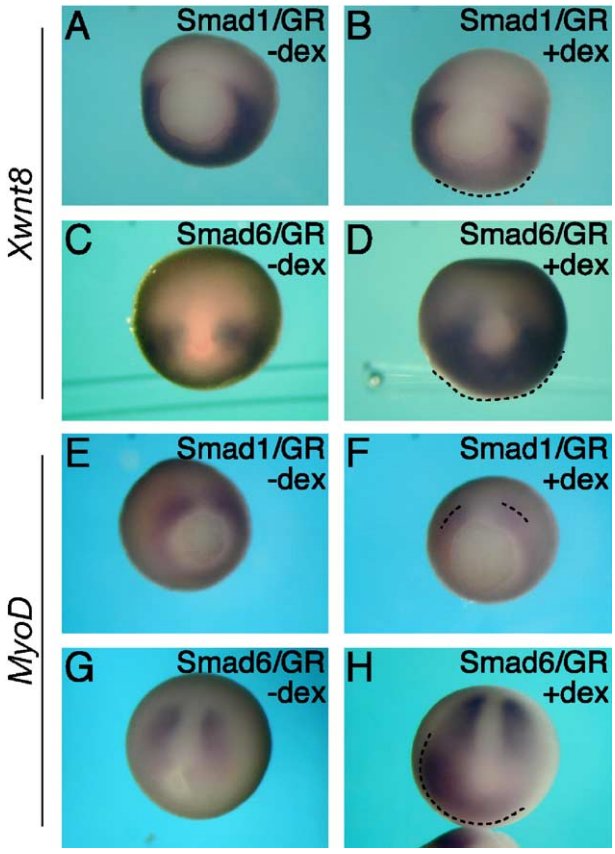

Fig. 6. BMP signaling performs a mesodermal patterning role during gastrulation. Embryos were injected with Smad1/GR (A,B,E,F) or Smad6/GR mRNA (C,D,G,H). The Smad1/GR-injected embryos were exposed to dex at st. 11 and fixed at st. 11.5 and the Smad6/GR-injected embryos were treated with dex at st. 11.5 and fixed at st. 12–12.5. The embryos were processed for in situ hybridization with *Xwnt8* (A–D) or *MyoD* (E–H) specific probes to determine changes in their patterns of expression. The regions exhibiting changes from the normal expression patterns are marked by dashed lines.

inducing gene *twin* in the ventral marginal zone (not shown). These results suggest that BMP2 is the main negative regulator of organizer-specific gene activation during blastula stages.

Discussion

Endogenous BMP signaling from MBT to gastrulation

Overexpression of BMP or experimental activation of the BMP signaling pathway in *Xenopus* embryos usually results in complex phenotypes arising from the activity of this growth factor family in numerous morphogenetic processes. Specific functions have been tentatively attributed to individual BMP ligands based on temporal and spatial patterns of expression. During blastula and early gastrula stages, three BMP family members are expressed, *BMP2*, *BMP4* and *BMP7* (Dale and Jones, 1999). *BMP2* and *BMP7* exhibit high maternal transcript levels that decrease towards gastrulation (Hemmati-Brivanlou and Thomsen, 1995; Nishimatsu et al., 1992), while elevated *BMP4* transcript levels are the result of zygotic transcription from MBT to the onset of gastrulation (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). During these stages, *BMP2* and *BMP4* transcripts are widely distributed throughout the embryo (Clement et al., 1995; Fainsod et al., 1994), making the analysis of localized BMP signaling very complicated. BMP signaling is activated soon after MBT throughout the embryo in a transcription-independent manner as evidenced by the appearance of phosphorylated BMP-specific R-Smads, and subsequently becomes ventrally localized as gastrulation progresses (Faure et al., 2000; Kurata et al., 2001; Schohl and Fagotto, 2002). The widespread activation of BMP signaling during blastula stages is in agreement with the suggested anti-organizer activity of this signal, while the subsequent ventral localization is consistent with the mesoderm patterning role. The phosphorylated R-Smad approach detects activation of the pathway but not its function. The inducible BMP-specific R- and I-Smad constructs complement this approach by allowing temporal manipulation of BMP activities. We identified distinct time windows for the anti-organizer and ventral patterning BMP-related activities. The use of the inducible Smad constructs also permits the manipulation of BMP signaling during later morphogenetic processes, thus allowing the embryo to develop normally until the desired developmental window as it has been used to study the role of BMP signaling in erythropoiesis (Schmerer and Evans, 2003).

Zebrafish embryos lacking maternal and zygotic transcripts of *Alk8*, a type I BMP receptor, exhibit strong dorsalization while loss of only the zygotic contribution results in mild dorsalization, showing the requirement for early and post-MBT BMP signaling with differing phenotypic outcomes (Bauer et al., 2001; Mintzer et al., 2001). Also, analysis of the zebrafish BMP-specific R-Smads, *Smad1* and *Smad5* genes, has revealed temporal differences in their transcription patterns which suggest distinct roles (Dick et al., 1999). *Smad5* is required for BMP signaling during late blastula but not during gastrula (Hild et al.,

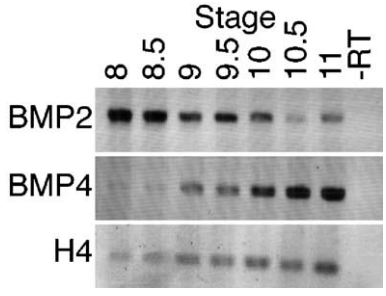

Fig. 7. Temporal pattern of *BMP2* and *BMP4* expression. RNA was prepared from normal embryos at different stages. The expression of *BMP2* and *BMP4* in the different samples was determined by RT-PCR with specific primers. The level of histone H4 expression was determined as a loading control.

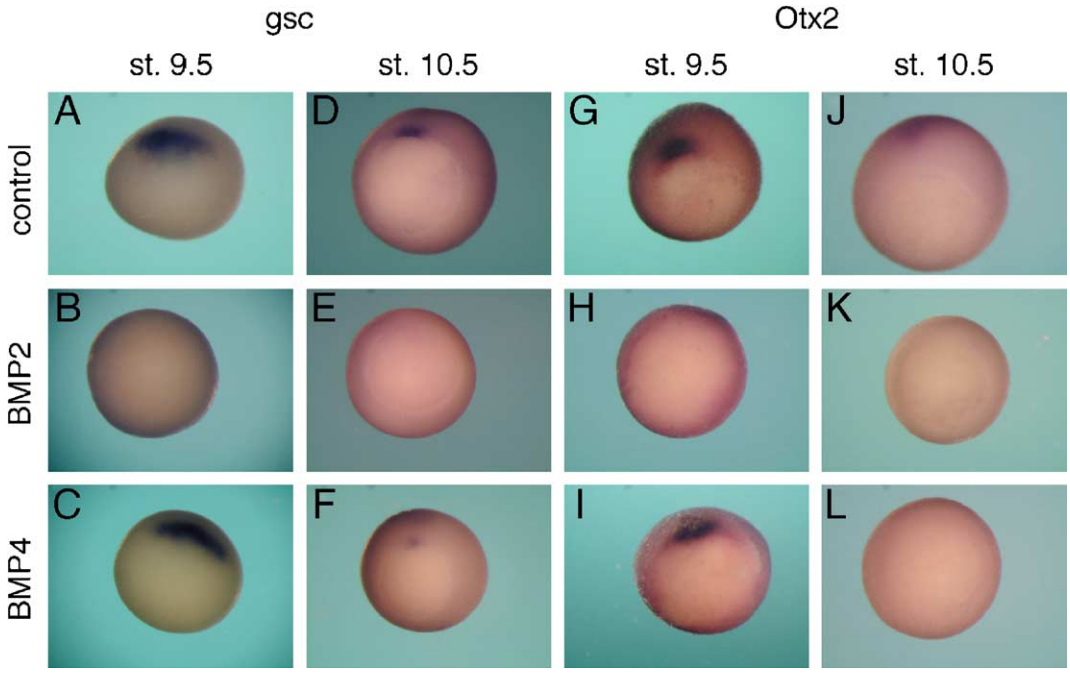

Fig. 8. BMP2 and BMP4 regulate organizer-specific expression at different times during embryogenesis. Embryos were injected with *BMP2* (B,E,H,K) and *BMP4* (C,F,I,L) mRNA and subjected to in situ hybridization analysis with *gsc* (A–F) and *Otx2* (G–L) specific probes at stage 9.5 (A–C, G–I) and stage 10.5 (D–F, J–L).

1999; Kramer et al., 2002). These observations suggest a model where a combination of different BMP factors performing the signaling and restriction in the competence of the responding tissues result in different developmental outcomes.

BMP signaling and the formation of Spemann's organizer

Inhibition of BMP signaling in the prospective ventral marginal zone by various approaches results in the formation of secondary axes in *Xenopus* embryos (Frisch and Wright, 1998; Hata et al., 1998; Hawley et al., 1995; Sasai et al., 1994; Suzuki et al., 1994), suggesting that BMP signaling plays a negative regulatory role on the formation of ectopic organizer-like structures in the embryo. The BMP downstream genes, *Xvex1* and *Xvent2*/*Vox*, are also widely expressed in blastula stage embryos, and have been shown to function as direct repressors of organizer-specific gene expression prior to gastrulation. These observations propose the establishment of a BMP-dependent inhibitory threshold of organizer formation (Melby et al., 1999; Shapira et al., 2000). The physiological relevance of this threshold might be linked to low levels of organizer-inducing Wnt pathway signal present throughout blastula stage embryos requiring active inhibition of potential ectopic organizer formation (Schohl and Fagotto, 2002).

The zygotic phase in Spemann's organizer establishment gets underway from MBT to early gastrula as demonstrated by timed activation of axis-inducing constructs (Darken and Wilson, 2001; Hamilton et al., 2001; Kodjabachian and Lemaire, 2001; Levy et al., 2002; Melby et al., 1999; Shapira et al., 2000). Timed activation of all three BMP-specific *Smad* constructs induced ventralization, dorsalization, or secondary axes with the same temporal restriction as axis induction, indicating that, from MBT to early gastrula, two different signals oppose each other: one acting to induce the organizer (Wnt) and a second one acting to restrict the organizer formation (BMP). During this time window, the organizer is in a labile state and BMP signaling can repress the formation of the embryonic organizer resulting in the "ventralized" phenotype. *Bozozok/dharma* promotes the formation of the embryonic shield, the zebrafish organizer, by antagonizing the expression of the BMP pathway genes *zbmp2b*, *vox/vega1*, and *vent/vega2* (Fekany et al., 1999; Imai et al., 2001; Kawahara et al., 2000; Koos and Ho, 1999; Leung et al., 2003; Melby et al., 2000). This regulatory interaction, antagonism of the BMP pathway, takes place also prior to gastrulation. The temporal overlap between phenotypic dorsalization and secondary axis induction suggests that Smad6, by blocking BMP signaling, might allow the marginal zone to transform into an organizer-type tissue in a manner reminiscent of LiCl treatment (Kao and Elinson, 1988; Klein and Melton, 1996). LiCl in turn has been shown to be able to dorsalize ventral marginal zone explants only when applied during the same developmental window, exhibiting efficient and weak dorsalization during blastula and gastrula stages, respectively (Lettice and Slack, 1993).

BMP overexpression like UV-treatment can promote the formation of ventral structures at the expense of dorsal ones in *Xenopus* embryos (Clement et al., 1995; Dale et al., 1992; Gerhart et al., 1989; Jones et al., 1992). Both treatments

have been linked through the UV-induced precocious up-regulation of *BMP4* transcription during blastula stages (Fainsod et al., 1994), and the observation that inhibition of BMP signaling can rescue UV-ventralized embryos (Sasai et al., 1994; Smith and Harland, 1992; Steinbeisser et al., 1995). Together, it appears that early BMP signaling performs an "anti-organizer" activity, which serves to restrict the formation of the organizer to only one, at the site of maximal *β-catenin* nuclear localization (Heasman, 1997; Shapira et al., 2000).

Overexpression of *BMP2* efficiently inhibited organizer-specific gene expression while BMP4 had only limited repressive activity. In embryos "ventralized" by *BMP4* overexpression, activation of organizer-specific genes initiates normally but their expression is repressed soon thereafter, suggesting a regulatory role in the maintenance of their expression (Fainsod et al., 1994; Jones et al., 1996; Steinbeisser et al., 1995). In contrast, BMP2 overexpression prevents organizer-specific genes from being expressed after the midblastula transition. These observations suggest that maternal BMP2 might antagonize the formation of the organizer from MBT and may be the main ligand repressing organizer formation along the marginal zone. The developmental effects together with the pattern of *BMP4* expression on the other hand, place BMP4 as an important element providing ventral mesodermal identities and this activity takes place during gastrulation (Jones et al., 1996).

BMP signaling in dorsoventral patterning of the mesoderm

Activation of the BMP-specific Smad proteins from early gastrulation onwards resulted in no apparent developmental malformation of the embryos but induced changes in the pattern of expression of marginal zone genes. The pattern of expression of *Xnot2* (dorsal), *MyoD* (lateral), and *Wnt8* (ventral) was affected by manipulation of the BMP signal during gastrula. These observations suggest the induction of dorsal–ventral patterning defects of the mesoderm. *BMP4* is expressed from the onset of gastrulation in the ventral and lateral marginal zone and is excluded from the newly formed Spemann's organizer (Fainsod et al., 1994; Schmidt et al., 1995), and BMP signaling is active in the same region (Faure et al., 2000; Kurata et al., 2001; Schohl and Fagotto, 2002). This activity together with the expression of BMP4 is in agreement with the proposed role of BMPs, particularly *BMP4* in promoting ventral fates in the mesoderm (Dale et al., 1992; Eimon and Harland, 1999; Fainsod et al., 1994; Jones et al., 1996; Jones et al., 1992; Schmidt et al., 1995; Steinbeisser et al., 1995), functioning as a morphogen in dorsoventral patterning of the *Xenopus* mesoderm (Dosch et al., 1997; Jones and Smith, 1998; Marom et al., 1999).

Judging from the phenotypes obtained from timed activation of the Smad proteins, ventralization of the mesoderm during gastrulation, although very important for the outcome of the embryo, results in externally normal-looking embryos. Similarly, localized misexpression of the *Xnot2* gene also results in mesodermal patterning effects (Gont et al., 1996). Dorsal overexpression of this gene results in enlargement of the notochord while ventral expression results in ectopic notochords, both at the expense of other mesodermal cells types. Embryos overexpressing *Xnot2* are externally normal looking (Gont et al., 1996). Therefore, abnormal dorsoventral patterning of the mesoderm can take place without changes in the DAI of the embryos in agreement with the timed manipulation of the BMP signal.

Negative regulation of organizer formation—a model

Secondary axis induction is one of the common assays employed to identify elements active in the formation of the embryonic organizer (Harland and Gerhart, 1997). Using this assay, it was determined that BMP signaling performs a negative regulatory role on organizer formation, and its inhibition creates a permissive region where an endogenous axis-inducing activity present throughout the marginal zone is relieved from its repression. Together with this study, the temporal competence of the ventral marginal zone to form an organizer and subsequently a secondary axis has been studied using inducible permissive (BMP inhibition) or instructive (activation of Wnt signaling and its targets) constructs (Darken and Wilson, 2001; Hamilton et al., 2001; Kodjabachian and Lemaire, 2001; Levy et al., 2002; Melby et al., 1999; Moon and Kimelman, 1998; Shapira et al., 2000). In all instances, the competence ended with the onset of gastrulation (Fig. 9, blue shaded area). Gastrulation, on

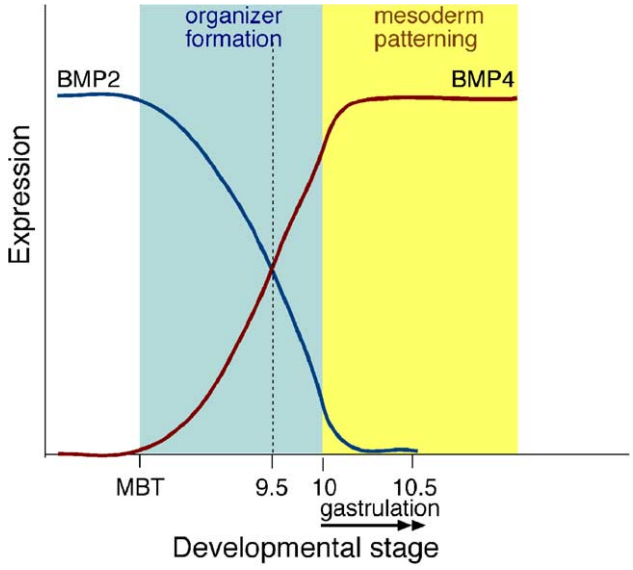

Fig. 9. The role of BMP signaling in the inhibition of organizer formation—a model. Diagrammatic representation of the changes in transcript levels between maternal *BMP2* transcript and zygotic *BMP4* mRNA. The developmental window when the marginal zone cells are competent to respond to organizer induction is shaded blue. Mesoderm patterning is shaded yellow. *BMP4* transcript levels increase zygotically before the competence for organizer induction ends.

the other hand, has been defined by indirect (Jones et al., 1996) and direct (this study) approaches as the developmental stage when the mesoderm undergoes patterning (Fig. 9, yellow shaded area). The distinction between organizer formation and mesoderm patterning can become complicated by the fact that the organizer itself subsequently functions in promoting dorsal mesoderm tissues and genes with axis-inducing potential play a role in mesodermal patterning (De Robertis et al., 2000). The use of the inducible Smad constructs allowed us to define functionally the onset of gastrulation as the transition from anti-organizer activity to a patterning role (this work), and it apparently marks the end of the neural induction window (Wawersik et al., 2005). It became clear that interference with Spemann's organizer formation results in severe developmental malformations normally known as "ventralization" or "dorsalization", while interference with the process of mesoderm patterning, although detectable with molecular markers, does not result in external phenotypes.

During the stages when ectopic organizers can be induced, BMP2 transcripts are present in high abundance (Hemmati-Brivanlou and Thomsen, 1995). Towards the end of the organizer-induction competence window, the *BMP2* transcripts are replaced by zygotic *BMP4* expression (Fig. 9). The replacement between abundant BMP ligands just before the end of the competence window permits both ligands, BMP2 and BMP4, to antagonize the formation of the organizer. On the other hand, it appears that BMP2 can prevent organizer-specific gene expression from its onset, while BMP4 can repress already active organizer genes probably as a result of its mesoderm patterning role in agreement with their temporal patterns of expression in the embryo. Therefore, BMP2 is suggested as the main BMP ligand active in the prevention of ectopic organizer formation along the marginal zone from MBT to the onset of gastrulation. *BMP4*, whose zygotic transcription increases from late blastula onwards, still inhibits the formation of the organizer by repressing the already active organizer-specific genes and subsequently performs a ventral patterning role along the mesoderm.

The spectrum of responses to BMP signals changes during embryogenesis. Different responses can be achieved either by competence changes of the responding tissue (Levy et al., 2002), by changes in components of the pathway (Dick et al., 1999), by diverse BMP ligands activating the pathway (Hemmati-Brivanlou and Thomsen, 1995; Nishimatsu et al., 1992), or by regulated processing of the ligands (Cui et al., 2001), among others. The patterns of *BMP* gene expression during blastula and gastrula support the suggestion that the actual BMP ligand involved in signaling might change. Also, the Smad proteins might also change as in zebrafish. We show that the forming organizer responds differently to BMP2 and BMP4 from MBT to the onset of gastrulation. During the same developmental stages, manipulation of BMP signaling loses its ability to antagonize or promote organizer formation, even though it continues to affect dorsal–ventral mesoderm patterning. Therefore, our results identify discrete BMP signaling functions separated in time, and suggest that these functions might be activated by different BMP ligands.

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